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Changes in glomerular epithelial cells induced by FGF2 and FGF2 neutralizing antibody in puromycin aminonucleoside nephropathy

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Changes in glomerular epithelial cells induced by FGF2 and FGF2 neutralizing antibody in puromycin aminonucleoside nephropathy. In the present study, two series of experiments were done with PAN nephropathy rats given fibroblast growth factor 2 (FGF2) or FGF2 neutralizing antibodies. In the first series of experiments, a dose of 10 μg of FGF2 (FGF2 group), 40 μg of an FGF2 neutralizing antibody (Anti-FGF2 group) or an equal volume of physiological saline (Control group) was administered for four days after PAN injection. Urinary protein increased more in the FGF2 group than in the other two groups. PCNA (+) glomerular cells were found in decreasing order in groups FGF2, Control and Anti-FGF2. Most of the PCNA (+) cells were podocytes and epithelial cells of Bowman's capsule. Staining for desmin, a marker of podocyte injury, was significantly reduced in the Anti-FGF2 group. Glomerular adhesive lesions were found in decreasing order in groups FGF2, Control and Anti-FGF2. The second series of experiments was designed to study the effects of FGF2 neutralizing antibody (40 μg for 5 days after PAN injection, in MoAb group) on severely damaged podocytes caused by repeated (two courses) injections in the PAN nephropathy rats. The results were the same as those in series 1. An increase in urinary protein excretion was observed in both groups, but on the 40th day, the level of proteinuria in the MoAb group decreased abruptly. It was observed that the MoAb group had few adhesive glomeruli compared to the IgG group (administration of mouse IgG) and the PCNA (+) epithelial cells of Bowman's capsule were also few. It was supposed that FGF2 would promote the formation of adhesive lesions by stimulating the proliferation of podocytes and epithelial cells of Bowman's capsule. Additionally, FGF2 itself was thought to impair podocytes because of the increasing desmin score and proteinuria.

The visceral epithelial cells or podocytes are the most differentiated cell type of the renal glomerulus. Under pathological conditions, unique changes occur in the cell architecture of podocytes. Among cells forming the glomerulus, podocytes do not readily proliferate under normal conditions. However, epithelial cells of Bowman's capsule (which have the same origin as podocytes during the embryonal period) show a comparatively higher replicative potential [1, 2]. Also, previous studies using rats with diabetic nephropathy [3], unilateral and subtotal nephrectomy [4, 5] and anti-GBM nephritis [6] showed neither evidence for podcoyte nuclear division nor an increase in the number of podoyctes per glomerular cross section. When podocytes are injured, they become detached from the glomerular basement membrane (GBM). Detachment of podocytes from the GBM at such sites, allows the epithelial cells of Bowman's capsule access to the GBM. This lesion is defined as early glomerular adhesion [7]. Such an early adhesion is generally not associated with sclerotic lesions in the corresponding tuft area. These lesions develop later. Proliferation of epithelial cells of Bowman's capsule is observed at the site of adhesion and is involved in their formation [4, 5, 8–12]. Therefore, it may be important that the factor should be mitogen for epithelial cells of Bowman's capsule in the early adhesive glomerular lesions. However, there have been few studies on the growth factors affecting proliferation of podocytes and epithelial cells of Bowman's capsule. It has been reported that fibroblast growth factor 2 (FGF2) has a proliferative effect on cultured glomerular epithelial cells [13]. Recent studies have shown that treatment of rats with FGF2 induces conspicuous structural changes in podocytes such as cell hypertrophy and widespread vacuolar formation. Segmental glomerulosclerosis with adhesive lesions was detected in these rats [14, 15]. We previously reported that the expression of FGF2 and FGF receptors was detected in damaged podocytes and epithelial cells of Bowman's capsule of rats with puromycin aminonucleoside (PAN) nephropathy [16]. Floege et al [17] suggest that released FGF2 from sublethal mesangial cell injury may be an important step in the initiation of mesangial proliferative glomerulonephritis. Therefore, we investigated the role of FGF2 in glomerular epithelial cell injury. In the present study, two series of experiments were done with PAN nephropathy rats given FGF2 and FGF2 neutralizing antibodies. The first series of experiments was intended to clarify the effect of FGF2 and FGF2 neutralizing antibodies on rats with PAN nephropathy after the induction of glomerular epithelial cell damage. The second series of experiments was designed to study the effect of FGF2 neutralizing antibody on more severely damaged glomerular epithelial cells of rats by repeated injection with PAN.

Methods

Animals

Male Wistar strain rats (Clea Japan Co., Osaka, Japan) weighing 110 g were used. The animals were housed in metabolic cages

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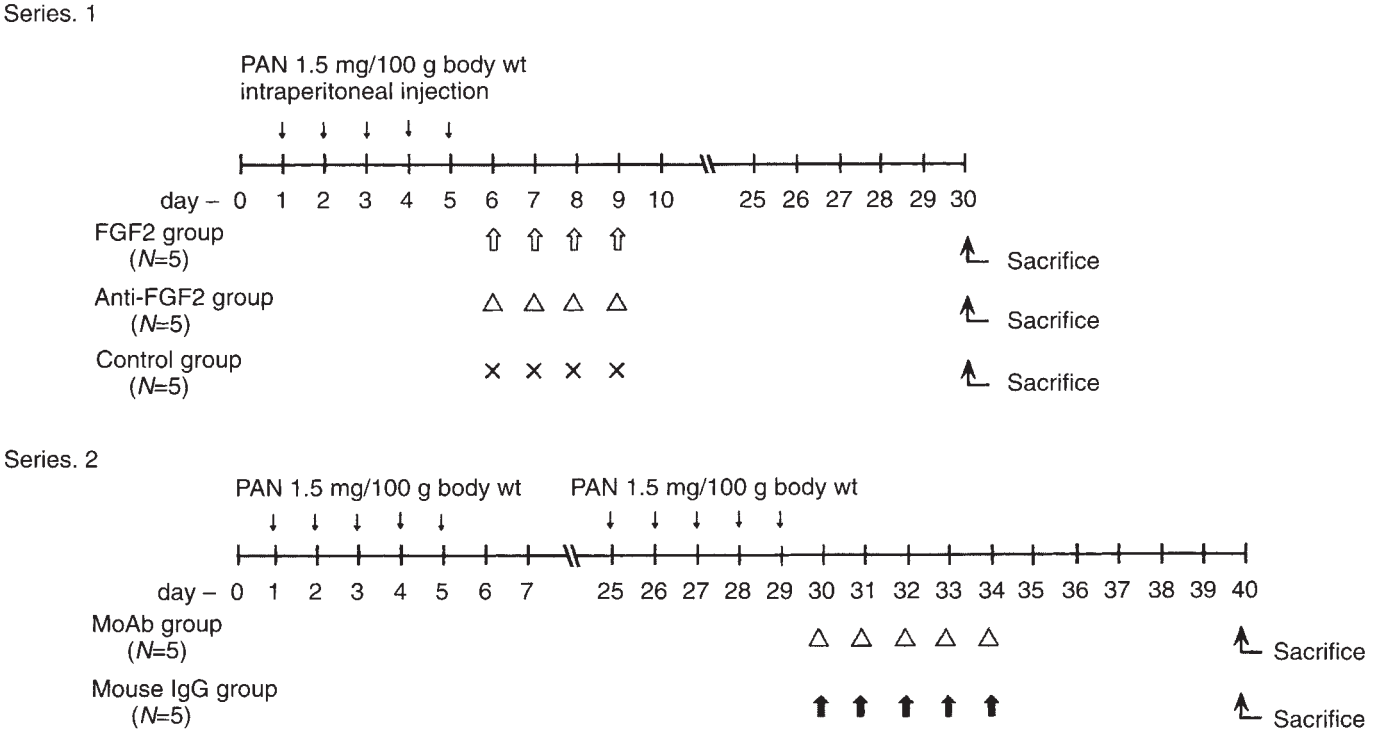

Fig. 1. *Experimental design.* Outline of the experimental design (series 1 and 2) showing various groups of rats studied. Symbols are: (⇧) 10 μg FGF2; (△) 40 μg neutralizing antibody of FGF2; (X) saline solution; (↑) 40 μg mouse IgG.

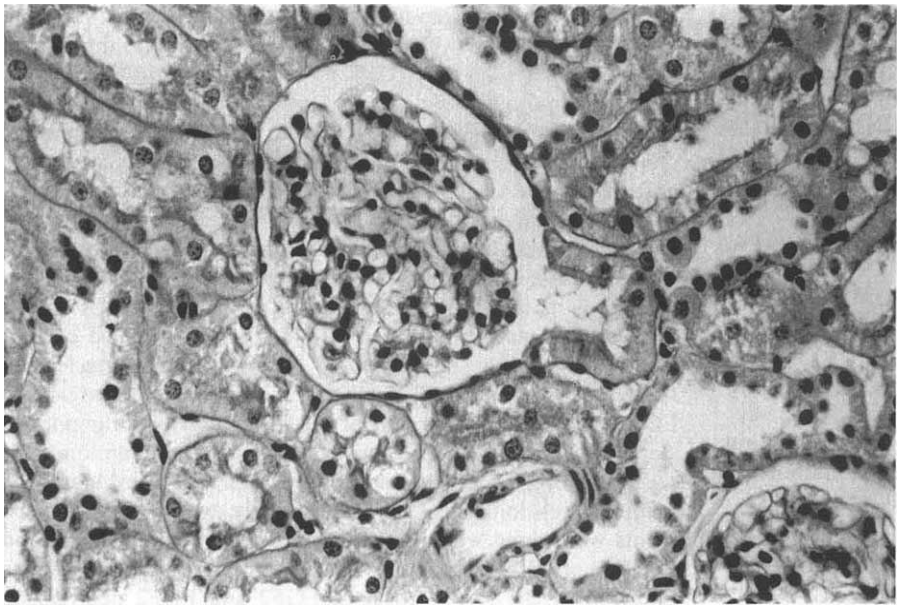

Fig. 2. *Normal Wistar rats were received 10 μg of recombinant FGF2 once a day for a period of four days.* No morphological abnormality of the glomerulus was found. PAS ×400.

and given food and water *ad libitum*. All surgery and all infusions were performed under general anesthesia with ether and additional injections of phenobarbital when necessary.

Series 1 experiments

PAN nephropathy was produced by intraperitoneal injections of PAN (1.5 mg/100 g body weight/day; Sigma Chemical Co., St. Louis, MO, USA) into rats for five days [18]. After PAN injection, 10 μg of recombinant FGF2 (Intergen Co., NY, USA) (N = 5, FGF2 group), 40 μg of FGF2-neutralizing antibody (clone 3H3) (N = 5, Anti-FGF2 group) or the equal volume of physiological saline (N = 5, Control group) was injected for four days into the tail vein, and the animals were sacrificed on the 30th day after the start of PAN administration (Fig. 1). Previous studies reported

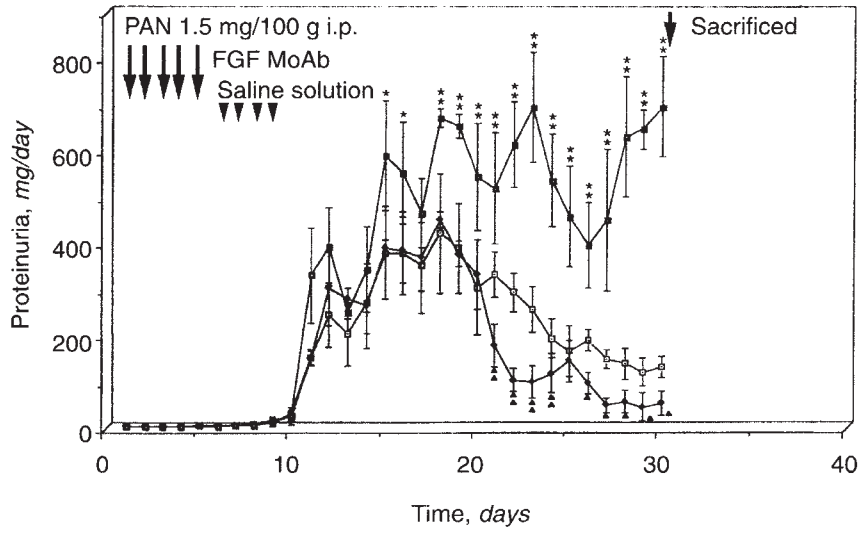

Fig. 3. *Daily urinary protein excretion of PAN rats following infusion of MoAb (Anti-FGF2 group), FGF2 (FGF2 group), saline solution (Control group).* All values are expressed as the mean ± SD. It is apparent that significant changes in the secretion of urinary protein took place on the 16th day, which increased more in the FGF2 group (□, $N = 5$) than in the other two groups. When compared, the level of urinary protein in the Anti-FGF2 group (◇, $N = 5$) was found to be lower than that in the Control group (⊡, $N = 5$) on the 21st day. $^{*}P < 0.05$, FGF2 group versus Anti-FGF2, $^{**}P < 0.01$, Control groups. ▲Anti-FGF2 group versus Control group.

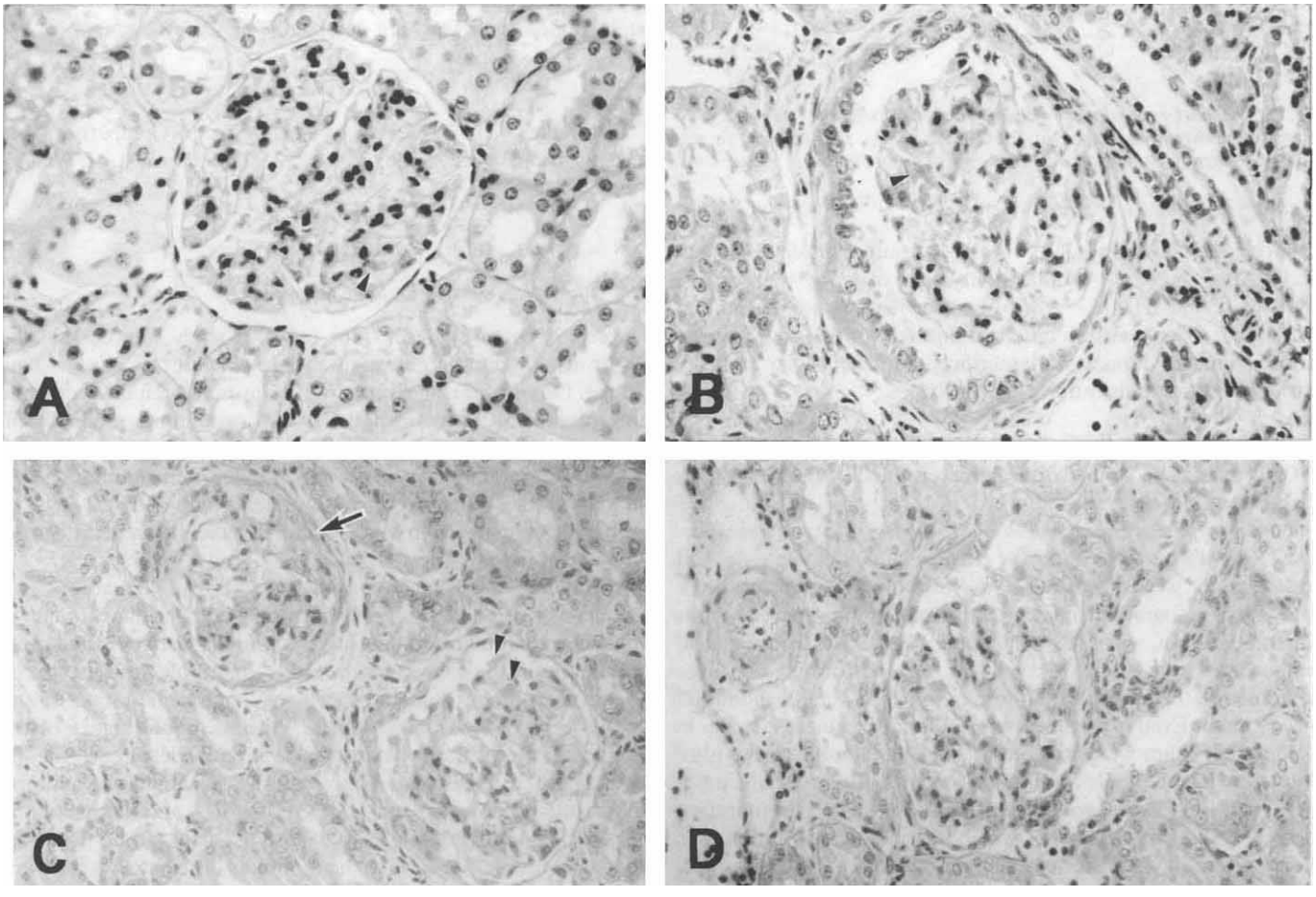

Fig. 4. A. *There are slight morphological abnormalities of the glomerulus of the Control group rats.* Cytoplasmic granules that a stained positive with PAS are present in podocytes (arrowhead). (PAS ×400). **B.** Cytoplasmic granules that stained positive with PAS are present in podocytes (arrowhead); hypertrophic and proliferative epithelial cells of Bowman's capsule (arrowheads) can be seen in the FGF2 group rats (PAS ×400). **C, D.** The proliferation of glomerular epithelial cells can be seen in the adhesive lesions in the FGF2 group rats (PAS ×400).

that clone 3H3 has an immuno-neutralizing activity for FGF2-induced proliferation of human umbilical vein endothelial cells [19]. Also, our study showed inhibition of FGF2-induced proliferation of cultured mesangial cell (data not shown).

Series 2 experiments

More frequent sclerotic lesions with adhesion were produced by intraperitoneal injection of 1.5 mg/100 g body wt of PAN into rats

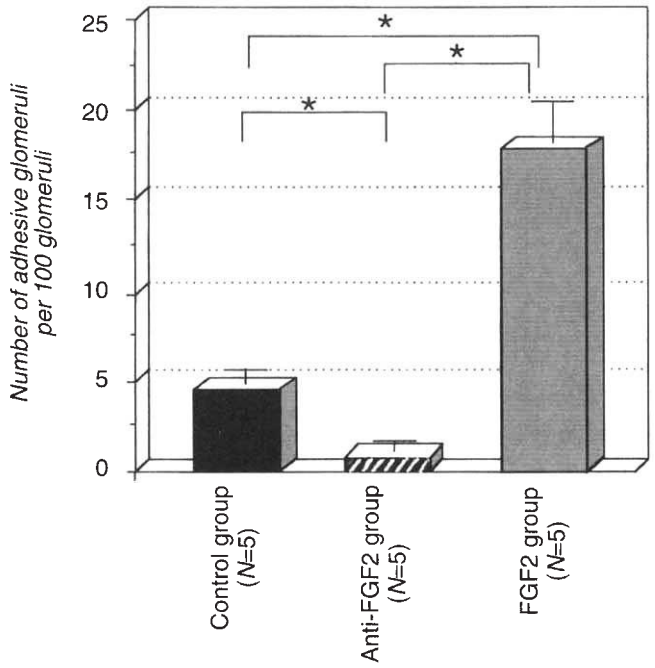

Fig. 5. *The number of adhesive glomeruli per 100 glomeruli.* The number of adhesive glomeruli is larger in the FGF2 group and smaller in the Anti-FGF2 group compared with the Control group. Data are mean ± SD.

for five consecutive days [18], on the first and 25th days, respectively. After the last PAN injection, 40 μg of FGF2-neutralizing antibody (clone 3H3; $N = 5$, MoAb group) or an equal volume of mouse IgG ($N = 5$, Group IgG) was injected for five days into the tail vein, and the animals were sacrificed on the 40th day after the start of PAN administration (Fig. 1).

Level of urinary protein

Daily urinary protein levels were determined by the Kingsbury-Clark's method.

Renal morphology and immunohistochemistry. Rats were perfused with 4% paraformaldehyde (PFA) via the left ventricle, and both kidneys were fixed. After dehydration, the kidneys were embedded in paraffin and thin sections were prepared. After deparaffinization, periodic acid-Schiff (PAS) or periodic acid methenamine silver (PAM) staining was performed on these sections, and the number of adhesive glomeruli per 100 glomeruli was counted. Even if there was more than one adhesive lesion in a glomerulus, they were counted as a single adhesive glomerulus. The following antibodies were used for immunoperoxidase staining of 4% PFA fixed paraffin sections: PC10 (a cell proliferation marker; Dako Co., Denmark), which is a monoclonal antibody to the proliferating cell nuclear antigen (PCNA), ED1 (Serotec, Oxford, UK), a monoclonal antibody to monocyte/macrophage, which checked presence of these cells in glomeruli, and D33 (Dako), a monoclonal antibody to desmin that is detected in podocyte injury [20]. The sections were treated with 0.3% hydrogen peroxidase in methanol for 30 minutes at room temperature to inactivate endogenous peroxidase. Then they were immersed in non-immunohorse serum for 30 minutes, washed in 0.02 M phosphate-buffer saline (pH 7.2), and were made to react with primary monoclonal antibodies for two hours at room temperature. After washing in phosphate-buffer saline, bound antibodies were detected using the ABC kit (Vector Co., Barlingame, USA). The bound antibodies were detected using 3,3-diaminobenzidine tetrahydrochloride (DAB) in Tris-buffer saline containing 0.02% hydrogen peroxide for 5 to 10 minutes. The number of PCNA (+) and ED1 (+) cells along with 50 glomeruli were observed. Glomerular desmin expression was evaluated semiquantitatively in four grades: Grade 0 (0 to 5%), Grade 1 (5 to 25%), Grade 2 (25 to 75%), Grade 4 (> 75%), depending on the appearance of positive staining.

Control study. Normal Wistar strain rats were divided into four groups, which received either 10 g of recombinant FGF2 ($N = 2$; Intergen Co., NY, USA), 40 g of FGF2-neutralizing antibody ($N = 2$; clone 3H3), the equal volume of mouse IgG ($N = 2$), or the equal volume of physiological saline ($N = 2$), respectively, once a day for a period of four days. Every day 24-hour urinary protein excretion was checked. Twenty-five days after the first day of injection, these rats were sacrificed. The four groups had no significant difference in the 24-hr urinary protein excretion and PCNA (+) glomerular cells. Adhesive glomeruli and desmin stained glomeruli were not detected (Fig. 2).

Statistical analysis

All values were expressed as the mean SD. Comparisons between groups were made using the Student's t-test or Welch's t-test.

Results

Series 1 experiments

Level of urinary protein. Our previous study showed that the 24-hr urinary protein excretion of normal adult rats was less than 10 mg. Onset of massive proteinuria was noted ten days after the intraperitoneal injection of PAN. This proteinuria gradually increased. Especially significant differences in the increase of urinary protein were observed on the 16th day, being more in group F than in the other two groups. When compared, the level of urinary protein in group M was found to be less than that of group C on the 21st day (Fig. 3).

Renal morphology and immunohistochemistry. The morphological changes of podocytes (such as cytoplasmic vacuolar formation and accumulation of PAS positive materials) were detected more frequently in the FGF2 group. Other morphological changes in the glomeruli consisted of hypertrophy and proliferation of epithelial cells of Bowman's capsule. Also, a mitotic figure of the epithelial cells of Bowman's capsule was easily detected at the site of glomerular adhesive lesions (Fig. 4). The number of adhesive glomeruli was higher in the FGF2 group and lower in the Anti-FGF2 group compared to the Control group (Fig. 5). Adhesive lesions appeared to be randomly distributed in the three groups. The PCNA (+) glomerular cells were mainly podocytes, and the number of epithelial cells of Bowman's capsule in the FGF2 group was higher than in the other two groups (Fig. 6A). There was no difference in the number of ED1 (+) cells in glomeruli in among the three groups. They were found exclusively in the mesangial areas and capillary lumens (Fig. 6B). These epithelial cells of Bowman's capsule were detected not only in adhesive lesions, but also in other places of the Bowman's capsule. The desmin score of glomeruli was lower in the Anti-FGF2 group

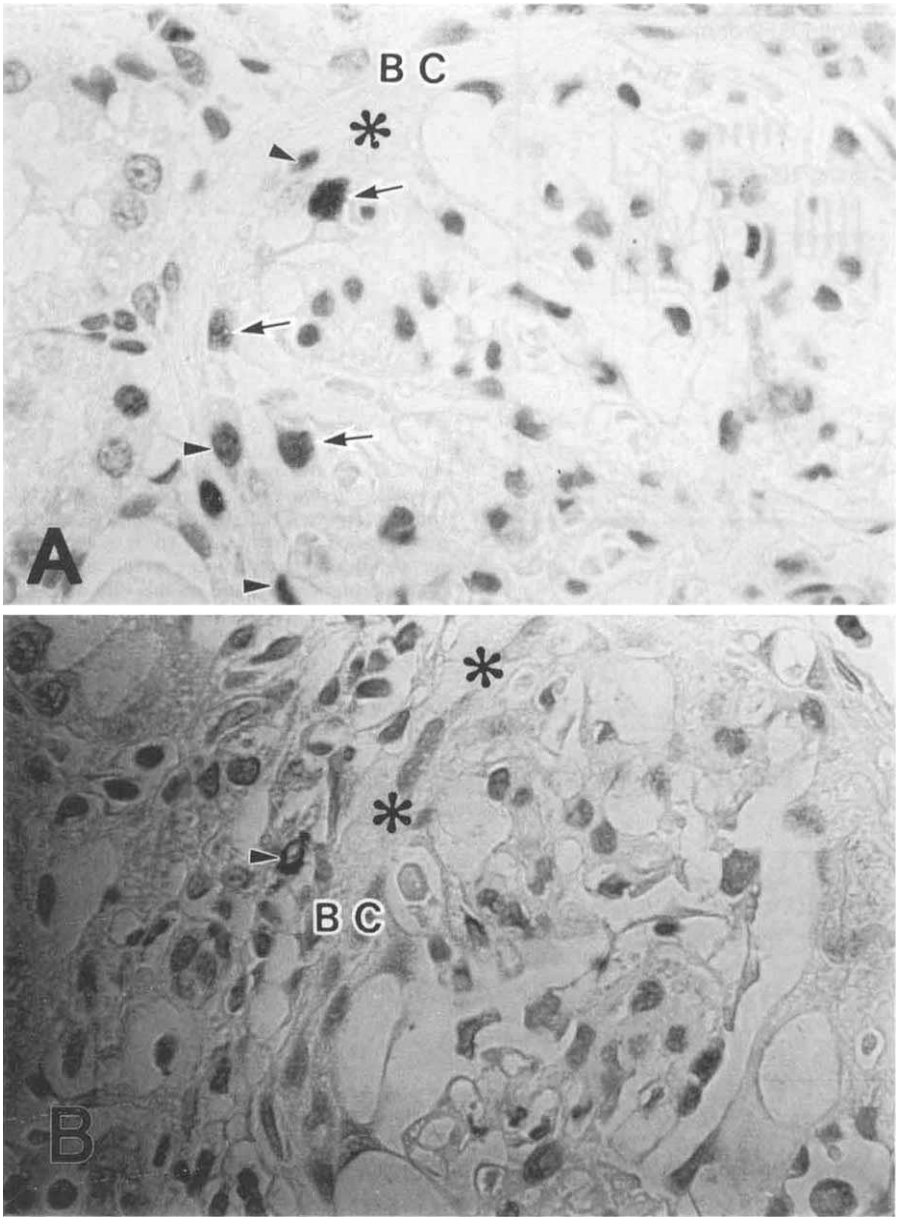

Fig. 6. A. *PCNA (+) cells are mainly podocytes (arrows) and epithelial cells of Bowman's capsule (arrowheads).* Asterisk, site of adhesive lesion; BC, Bowman's capsule (×1000). **B.** Site of adhesive lesion (asterisks) showing no participation of ED1 (+) cells, however, ED1 (+) cells (arrowhead) are present in the periglomerular interstitium (×1000).

than in the other two groups; however, no significant difference was noted between the Control and FGF2 groups (Table 1).

Series 2 experiments

Level of urinary protein. An increase in urinary protein excretion was observed in both groups, but on the 40th day, the level of proteinuria in the MoAb group decreased abruptly (Fig. 7).

Renal morphology and immunohistochemistry. In the same way as in series 1, the MoAb group had fewer adhesive glomeruli than the IgG group (Figs. 8 and 9). The frequency of PCNA (+) glomerular cells and epithelial cells of Bowman's capsule was also higher in the MoAb group than in the IgG group. However, there was no difference in the number of ED1 (+) cells. Also, the desmin score of glomeruli was lower in the MoAb group than in the IgG group (Fig. 10A, B and Table 2).

Discussion

This study showed that intravenous injection of FGF2 markedly increased the glomerular podocyte injury and proliferation of epithelial cells of Bowman's capsule in PAN nephropathy. The previous study reported that FGF2 rapidly cleared up from serum after intravenous injections and was deposited to a greater extent in solid organs than in blood vessel walls. Deposition was greatest in the kidney followed by the liver, and spleen and was substantially smaller in the heart and lung [21]. The present data as well as recent *in vivo* and *in vitro* findings suggested that FGF2 might be one of the factors involved. FGF is a member of a family of polypeptide growth factors (of which nine have been identified to date), which in turn are capable of being bound to any of the components of the tyrosine kinase receptors (4 of which have

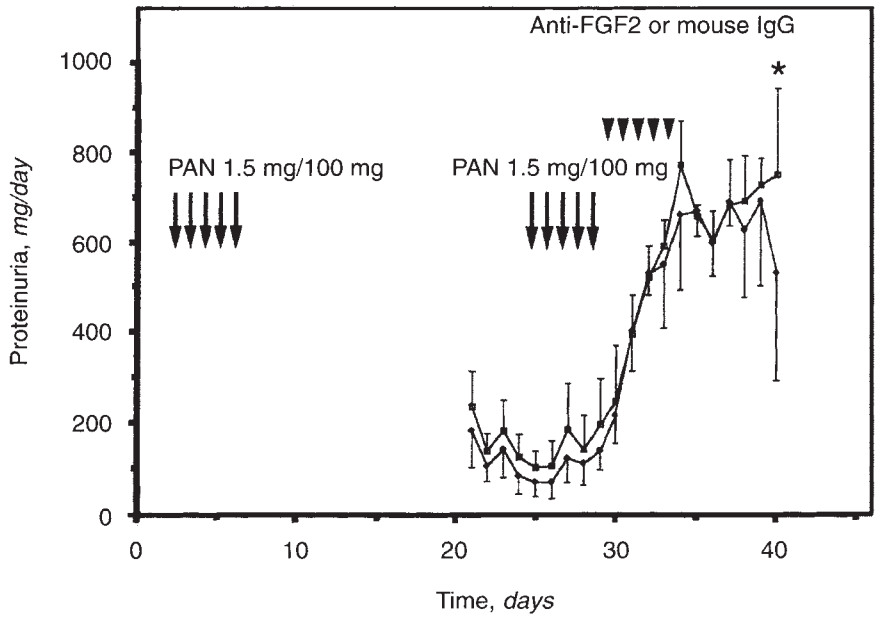

Fig. 7. *Daily urinary protein excretion of PAN rats following infusion of either MoAb (◇, MoAb group) or mouse IgG (□, IgG group).* All values are expressed as the mean ± SD. It is apparent that significant changes in the secretion of urinary protein took place on the 40th day. $^{*}P < 0.05$.

Table 1. Histological evaluation of series 1 study

	Control group ($N = 5$)	Anti-FGF 2 group ($N = 5$)	FGF 2 group ($N = 5$)
PCNA(+) glomerular cells	65.2 ± 27.3	26.2 ± 12.1	175.0 ± 57.2
PCNA(+) epithelial cells of Bowman's capsule	7.6 ± 2.5	4.1 ± 1.2	35.2 ± 12.3
ED 1(+) cells	7.0 ± 0.7	1.6 ± 0.9	2.2 ± 0.8
Desmin score	91.8 ± 12.1	21.3 ± 3.5	87.0 ± 21.0

The number of PCNA (+) glomerular cells, epithelial cells of Bowman's capsule, ED1 (+) cells and desmin score per 50 glomeruli.
Data are means ± SD.
[a] $P < 0.05$; [b] $P < 0.01$

Table 2. Histological evaluation of series 2 study

	MoAb group ($N = 5$)	IgG group ($N = 5$)
PCNA(+) glomerular cells	186.6 ± 5.7	253.8 ± 34.5
PCNA(+) epithelial cells of Bowman's capsule	25.2 ± 10.8	49.3 ± 15.8
ED 1(+) cells	2.6 ± 1.1	2.2 ± 0.8
Desmin score	101.0 ± 2.9	119.8 ± 11.2

The number of PCNA (+) glomerular cells, epithelial cells of Bowman's capsule, ED1 (+) cells and desmin score per 50 glomeruli.
Data are means ± SD.
[a] $P < 0.05$

been identified to date) [22]. One of these growth factors, FGF2, has received particular attention as a potential mediator of glomerular injury. The role of FGF2 in *in vivo* models of wound healing, angiogenesis, and cardiovascular development has been demonstrated. A number of glomerular cell types, such as mesangial cells [23], endothelial cells [24] and visceral epithelial cells [13], can be stimulated to proliferate in response to exposure to FGF2. This implies that each of these cell types bears specific FGF receptors. In our previous studies, FGF2 protein was found in the normal rat glomeruli, but its mRNA was not detected. However, FGFR protein and FGFR1-4 mRNAs were present in the collecting ducts and distal tubules [25, 26]. FGF2 expression was observed in podocytes and epithelial cells of Bowman's capsule of PAN nephropathy rats [16]. FGF2 emits no signal before secretion, so the mechanism of its release is unclear. In cell injury, FGF2 is released from its cytosolic storage sites through plasma membrane disruption [27]. Extracellularly, it is bound to heparan sulfate proteoglycans where it may be released by heparin and heparitinases [28]. Disruption of the cytoplasm of damaged podocytes was observed under an electron microscope (data not shown), suggesting that FGF2 may have a paracrine influence on the proliferation of epithelial cells of Bowman's capsule. Since FGF2 mRNA is present in the epithelial cells of Bowman's capsule, an autocrine influence cannot be denied. Although no proliferative action of FGF2 is observed in normal glomeruli, such an effect on damaged mesangial cells has already been reported [29].

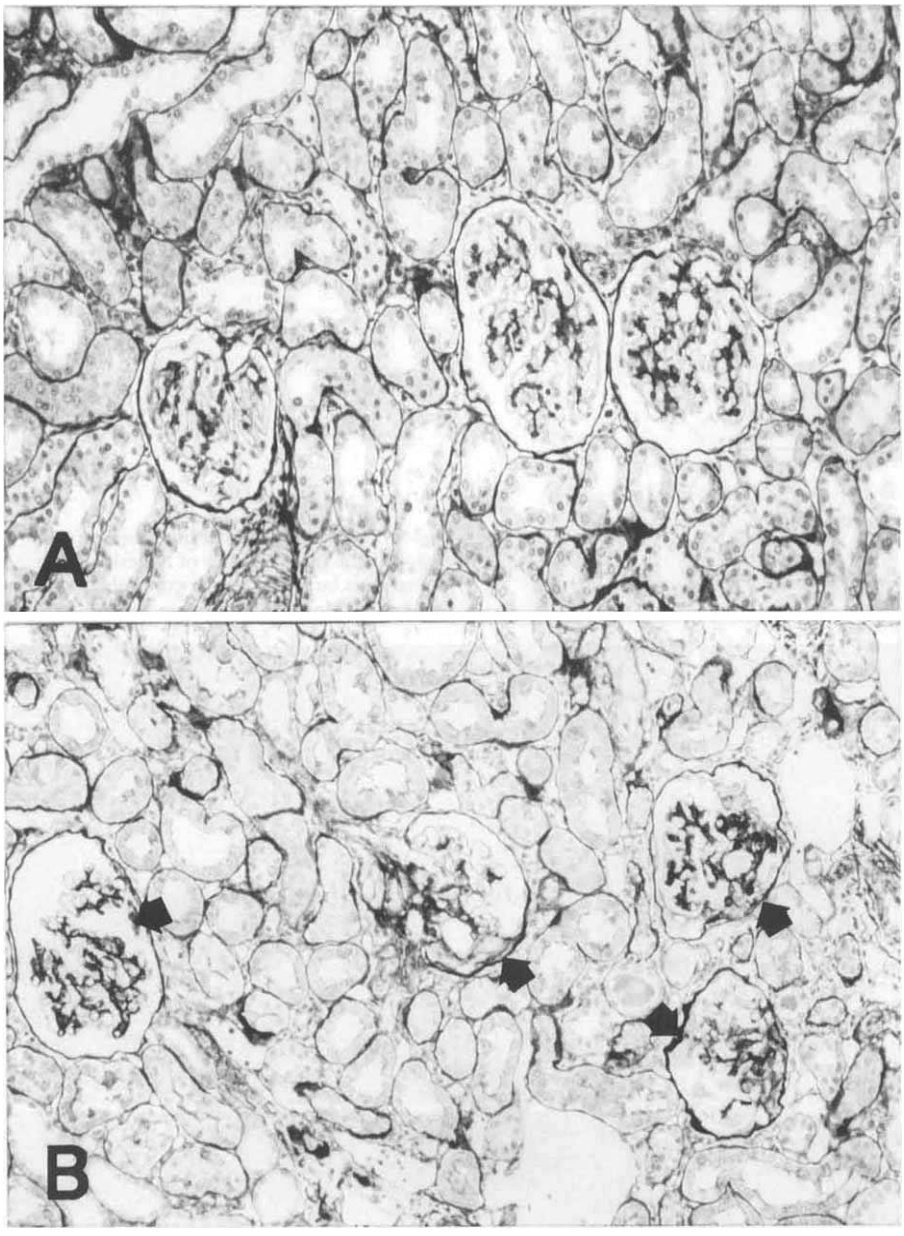

Fig. 8. *It is observed that the MoAb group (B) has fewer adhesive glomeruli (arrows) than the IgG group (A).* (PAM ×200).

In the present study, many PCNA (+) podocytes and epithelial cells of Bowman's capsule were observed when FGF2 was injected following PAN, and the PCNA (+) cells decreased when an FGF2-neutralizing antibody was injected. No monocyte/macrophage was found in adhesive lesions either. This result was considered to be related to FGFR mRNA expression by podocytes and epithelial cells of Bowman's capsule, suggesting an *in situ* hybridization [17]. However, further detailed studies are necessary for more clear and detailed understanding of the role of FGFR1-4. PCNA (+) podocytes showed morphological abnormalities, such as vacuolization and accumulation of PAS-positive materials in the cytoplasm. Since the desmin score, (a marker for damaged podocytes), was reduced by administration of the FGF2-neutralizing antibody, there is a possibility that FGF2 may play a role in podocyte damage. Some evidence suggests that nuclear division in podocytes was not followed by complete cell division. Kriz et al recently did not observe an increase in the absolute number of podocytes despite nuclear division in podocytes after long-term, high-dose administration of FGF2 to rats [15]. The FGF2-induced podocyte changes had severe structural abnormalities, which probably showed the important role of podocytes in maintaining the glomerular permeselectivity [30]. This indicated that FGF2 injection also increased the occurrence of proteinuria. Also, it has been suggested that FGF2 might become relevant in those podocytes that have more sensitivity to FGF2 due to an up-regulation of FGF2 receptors. FGF2 did not induce proliferation of glomerular cells in normal rats. Furthermore, Floege et al [31, 32] reported that rats receiving a subnephrotogenic dose of Heymann serum develop glomerulosclerosis in the additional treatment with small doses of FGF2. Recently the development of

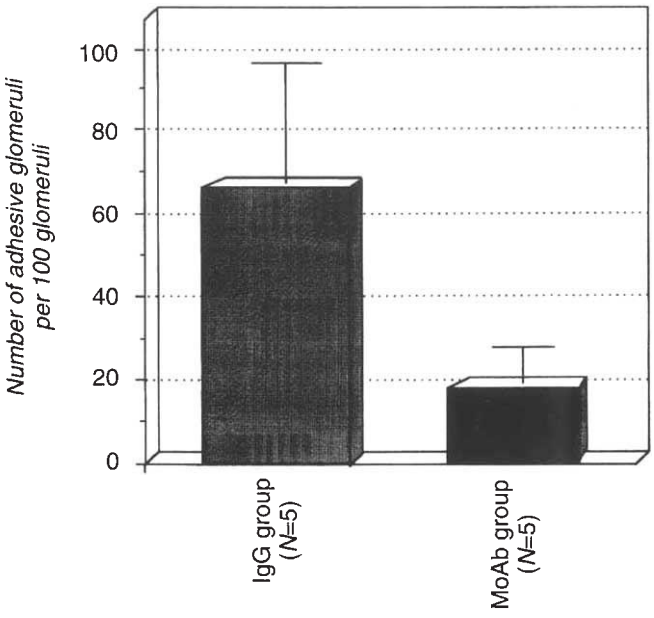

Fig. 9. *The number of adhesive glomeruli per 100 glomeruli.* The number of adhesive glomeruli is larger in MoAb group than in the IgG group. Mean ± SD.

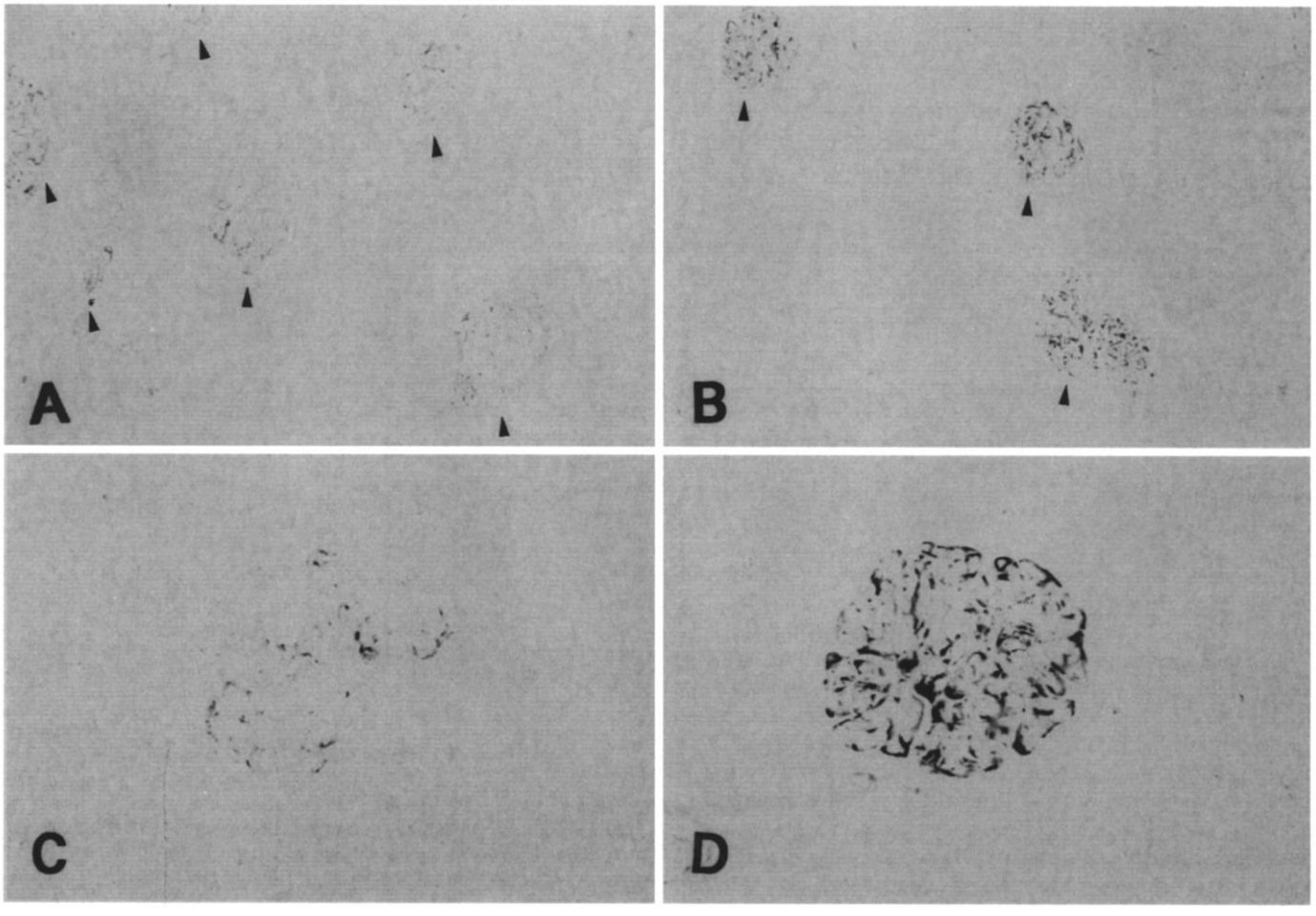

Fig. 10. *Desmin score of glomeruli (arrows) was lower in the MoAb group (A, C) than in the IgG group (B, D).* A, B ×200, C, D ×400.

HIV-associated nephropathy has been linked to FGF2 [33]. HIV-nephropathy consists of rapidly progressing focal glomerulosclerosis characterized by severe podocyte lesions [34]. These indicate that FGF2 released from injured podocytes may act synergistically with other damaging mechanisms.

Moreover, *in vitro* data from a previous study showed that

FGF2 released from injured podocytes may act on the opposite epithelial cells of Bowman's capsule to induce their proliferation. Since FGF2 administration caused an increase in PCNA (+) epithelial cells of Bowman's capsule whereas administration of neutralizing antibody inhibited the formation of adhesive glomeruli, proliferation of epithelial cells of Bowman's capsule may be suggestive of involvement of FGF2 in the formation of adhesive lesions. From these results, it can be assumed that FGF2 has a complex role in podocyte injury and induces subsequent responses in epithelial cells of Bowman's capsule.

Acknowledgments

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